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Determination of aflatoxin M_1 in milk and dairy products using high performance liquid chromatography-fluorescence with post column photochemical derivatization

Nor Shifa Shuib[a,b,*], Ahmad Makahleh[c], Salizawati Muhamad Salhimi[d], Bahruddin Saad[a,**]

[a] School of Chemical Sciences, Universiti Sains Malaysia, 11800, Pulau Pinang, Malaysia
[b] Mycotoxin Analytical Centre, Chemistry Department, Penang Branch, Jalan Tull, 10450, Pulau Pinang, Malaysia
[c] Department of Chemistry, University of Jordan, Amman, Jordan
[d] School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800, Pulau Pinang, Malaysia

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ABSTRACT

The determination of aflatoxin M_1 in milk using high performance liquid chromatography with photochemical post-column derivatization and fluorescence detection is described. The samples were first extracted and clean–up using the immunoaffinity AFLATEST column originally targeted for aflatoxins B_1, B_2, G_1 and G_2. The separation of aflatoxin M_1 were performed using C18 Hypersil gold (150 mm × 4.6 mm, 5 μm) column at 40 °C under isocratic elution. Fluorescence detector (FLD) was set at 360 nm and 440 nm as excitation and emission, respectively. The use of methanol to replace acetonitrile as the mobile phase resulted in ~67% peak area enhancement of AFM_1. The limit of detection (LOD) and quantification (LOQ) of the analytical method after post-column derivatization without evaporation/reconstitution with mobile phase was 0.0085 μg L^{-1} and 0.025 μg L^{-1} respectively. However, LOD and LOQ improved to 0.002 and 0.004 μg L^{-1} respectively with the addition of evaporation/reconstitution step. The method was statistically validated, showing linear response (R^2 > 0.999), good recoveries (85.2–107.0%) and relative standard deviations (RSD) were found to be ≤7%. The proposed method was applied to determine AFM_1 contamination in various types of milk and milk products. Only 2 samples were contaminated with aflatoxin M_1 (10% incidence). However, the contamination level is below the Malaysian and European legislation limits.

1. Introduction

Aflatoxin M_1 (AFM_1) is a metabolite produced by the hydroxylation of aflatoxin B_1 (AFB_1) in the liver of lactating animals and humans [1]. AFM_1 has been classified under Class 1 human carcinogen by the International Agency for Research on Cancer [2]. Due to its toxicity and its effect especially to children, the European Commision (EC) has set the maximum permitted level for AFM_1 at 0.05 μg kg^{-1} and 0.025 μg kg^{-1} in milk and infant formula, respectively [3]. As infants are more susceptible to mycotoxins than adults, ideally no mycotoxin including aflatoxin should be present in human food especially in the diet of infants. Therefore AFM_1 surveillance in dairy products requires sensitive analytical method to meet EC requirement. As such, validation at lower level is necessary, especially for infant formulas, to meet regulatory levels imposed by EC.

Methods for the determination of AFM_1 in milk includes thin layer chromatography (TLC), ELISA and HPLC [4,5]. Accurate quantitation of aflatoxins using TLC method is limited [5] while the ELISA method could provide false-positive results [6]. Reversed phase liquid chromatography (RP-HPLC) coupled with fluorescence detection (FLD) is currently the most commonly used method for the determination of AFM_1 in milk [5,7]. An important requirement in the determination of AFM_1 in infant food is to adopt sensitive methods. This is made possible by using mass spectrometry (MS) or subjecting the AFM_1 to a derivatization procedure. Although AFM_1 is a natural fluorescencing compound (do not require derivatization), several authors reported the use of either pre-column or post-column derivatization to enhance the AFM_1 sensitivity. Derivatization using suitable fluorophores (e.g., trifluoroacetic acid (TFA), bromination) are commonly applied for the determination of AFB_1 and AFG_1 in RP-HPLC methods [7–9]. This approach enhances

* Corresponding authors at: School of Chemical Sciences, Universiti Sains Malaysia, Pulau Pinang, 11800, Malaysia.
** Corresponding author.
E-mail addresses: nor_shifa@yahoo.com (N.S. Shuib), bahrud@usm.my (B. Saad).

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the natural fluorescence of AFB_1 and AFG_1 that contain unsaturated furan ring. In contrast, AFB_2 and AFG_2 (contain saturated furan ring) do not change their fluorescence properties as derivatization does not take place. As AFM_1 contains unsaturated furan ring, it can been derivatized either by pre-column or post-column approach. The pre-column approach normally involves the formation of the corresponding hemiacetals by TFA [7]. The post-column derivatization approach uses either bromination [7] in an electrochemical cell (Kobra Cell) or by the addition of bromide or pyridinium hydrobromide perbromide (PBPB) to the mobile phase [10]. Interestingly, the addition of cyclodextrin to the mobile phase was able to increase the sensitivity of AFM_1 [11].

These derivatization methods have several disadvantages including the use of toxic solvents (e.g., TFA, Bromine, Iodine), time consuming due to the solvent evaporation, limited stability and requires daily maintenance [12]. The use of post-column derivatization employing online irradiation system had been reported for the determination of AFB_1 and AFG_1 [13]. This method does not require toxic solvent and requires minimum maintenance as it is based on UV irradiation of the separated AFB_1 and AFG_1 before the detector. However, there is no report on the post-column online photochemical derivatization of AFM_1. In this paper, for the first time, photochemical derivatization technique was introduced to enhance the sensitivity of AFM_1 in milk and milk products. This activity is important in view of the lower detection limits (below EC requirements) that is required, particularly for infant formula ($0.025\ \mu g\ kg^{-1}$) [14].

2. Experimental

2.1. Chemicals and materials

AFM_1 standard was purchased from Sigma (St. Louis, MO, USA). Stock standard solution ($5\ \mu g\ L^{-1}$) was diluted with freshly prepared water:methanol (1:1, v/v). From this solution, a series of working standards (0.01, 0.05, 0.25, 0.5 and $1.0\ \mu g\ L^{-1}$) were prepared. Acetonitrile and methanol were of HPLC grade while all other chemicals were of analytical grade. Water was purified in a Milli-Q system on $18.2\ m\Omega\ cm^{-1}$. The immunoaffinity columns AFLATEST were obtained from VICAM (Watertown, MA, USA).

2.2. Sample preparation

2.2.1. Liquid milk

The procedure was based on the AOAC method [15] with some modifications. Fluid milk samples (10 mL) were centrifuged (4000 rpm) for 10 min to separate the fat before being applied to the immunoaffinity column. The column was washed twice with distilled water (10 mL), then eluted with methanol (500 μL instead of 1000 μL for the conventional method). In the conventional method, the methanol extracts are mixed with water (1:1, v/v).

2.2.1.1. Evaporation/reconstitution procedure.

The eluate was dried under nitrogen stream and reconstituted in 200 μL of methanol:water (1:1, v/v) and proceed for HPLC analysis.

2.2.2. Infant formula

Infant formula (10 mL) was suspended in warm water (100 mL) before the analyses. The liquid samples were centrifuged and the rest of the procedure was as stated for liquid milk (Section 2.2.1).

2.3. HPLC conditions

Chromatographic analyses were performed with Waters HPLC system (Waters, Milford, MA, USA) consisting of 2695 separation module connected to 2475 Multi-wavelength fluorescence detector. A post-column photochemical reactor (PHRED) with a mercury lamp (k = 254 nm) and a knitted reactor coil (5 m × 0.25 mm) (Watertown, MA, USA) was used.

Data acquisition was performed using Empower software (Waters). Separation was effected by using Hypersil Gold C18 analytical column (250 mm × 4.6 mm, 5 μm particle size) preceded by a C18 security guard cartridge (4.0 × 3.0 mm, 5 μm) from Phenomenex (Torrance, CA, USA).

The mobile phase was methanol:water (35:65, v/v). Excitation and emission wavelengths of the fluorescence detector (FLD) was set at 360 nm and 440 nm, respectively. The flow rate was $1.2\ mL\ min^{-1}$ and column temperature was maintained at 40 °C. Aliquots of sample extracts (25 μL) were injected.

2.4. Method validation

Validation of the method includes linearity, limits of detection (LOD) and quantification (LOQ), accuracy and precision.

LOD was determined as the lowest concentration that can be detected. LOQ was determined

as the lowest fortification level according to CEN/TR 16059 [16].

Accuracy was determined by the% recovery obtained from experiments conducted with blank milk samples spiked at three concentration levels (0.01, 0.05 and $0.5\ \mu g\ L^{-1}$). Each level was prepared in triplicate. The inter-day recovery was carried out for infant formula at $0.01\ \mu g\ L^{-1}$ for three different days (n = 9).

2.5. Statistical analysis

Statistical analysis was conducted using Statistical Package for Social Science (SPSS version 16). Analysis of variance (ANOVA) was conducted to determine differences in AFM_1 signal. The accepted confidence level required for significance was set at 95% ($P < 0.05$).

2.6. Application to real samples

UHT cow milk (10), human milk (3), infant formula (10) and goat milk (10) samples were analysed. They were purchased from local supermarkets and stores in Penang, Malaysia. Samples were stored at −20 °C until analysis.

3. Results and discussion

It is speculated that the derivatization is made possible by the presence of the unsaturated furan ring, similar to that of AFB_1 and AFB_2. Fig. 1 shows the post-column photochemical derivatization mechanisme of AFB_1 and AFM_1.

3.1. Optimization of the photochemical derivatization procedure

The HPLC mobile phase was assessed for its suitability for the photochemical derivatization. The most common mobile phase used in the determination of AFM_1 using chemical derivatization is water:methanol:acetonitrile (70:20:10, v/v/v) [7]. It must be pointed out that acetonitrile was used as it is compatible with TFA that was used for the derivatization process. However, the quenching of AFM_1 by acetonitrile has also been recognised [11]. Thus the substitution of acetonitrile by methanol in the HPLC mobile phase was evaluated. Different ratios of water:methanol (70:30, 65:35 and 60:40 v/v) were studied. It was found that among all the methanolic mixtures, 65:35 (v/v) produced sharper peaks, thus improving resolution. Therefore, water:methanol:acetonitrile (70:20:10, v/v/v) and water:methanol (65:35, v/v) were investigated with and without the online photochemical derivatization. The use of methanol:water (35:65, v/v)